International Journal of Research in Applied Sciences & Engineering (IJRASE)
Online ISSN: 2456-2920; Volume 1, Issue 1; January 2016 pp. 65-71

Computational Virtual Screening and Preliminary Cytotoxicity Assay of Novel Herbal Therapeutics on MCF-7 Cell Lines

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Abstract: **Breast cancer is the second most common type of cancer worldwide. The study mainly aims to screen herbal bioactive compounds against probable drug targets of Breast cancer through computational virtual screening and in vitro assay. From the literature survey the major receptors involved in breast cancer pathway were identified as probable drug targets. Fifty four herbal based bioactive compounds from various medicinal plants were screened by extensive literature survey and their drug likeliness and pharmacokinetics properties were computationally predicted. Molecular docking studies were carried out to understand the binding potential of the selected ligands towards the receptors. The best lead identified from docking analysis is subjected to preliminary in vitro assay against MCF-7 cell lines. Apigenin is identified as the best lead molecule from computational virtual screening. This phytoligand present naturally in *Petroselinum crispum,* Apium graveolens *and Matricaria chamomilla* available abundantly in the southern part of India. The MTT assay revealed that IC_{50} value of Apigenin was estimated to be 35µg. The current study suggests that herbal bioactive compounds especially Apigenin can be used as one of best inhibitors against various receptors of Breast cancer.**

Keywords: **Breast cancer, Apigenin, MCF-7 cell lines, Molecular docking, Drug likeness, IC_{50}, Metabolic pathways**

1. INTRODUCTION

Cancer as stated by various institutes as specialised in oncology studies is a collective term used for those diseases in which the cells that have become abnormal due to various causes divide uncontrollably and can invade other tissues. This abnormality may arise from diverse and complex factors which have only been partially understood [1]. Cancerous cells arise from various tissues such as skin, cartilage, bone, blood forming tissues, tissues of the brain, based on which they are grouped into categories [2]. Breast cancer stages involved may be determined by the epithelial cell differentiation in the mammary tissue and the markers that help in identifying these cells [3]. Tumorous lumps are set to arise from normal cells due to the accumulation of alterations in the genetic and epigenetic factors. Many molecular pathways and genes involved in normal cell functions were seen to be activated in cancer pathways [1].

India is one of the largest developing countries records a steadily rising incidence of breast cancer [4]. Epidemiology studies carried out in various Asian countries show that breast cancer as a disease may not be the same in India as that of western countries. The mortality rate of the patients affected is decreasing sporadically [5]. The most commonly used radiotherapy with or without hyperthermia (heating of tumour at high temperature) destroys normal as well as abnormal cells resulting in loss of metabolism and crucial cell functions [6]. Hormone replacement therapy carried out mostly in post-menopausal women which results high risk of venous or arterial thromboembolic disease including stroke and cardiovascular diseases [7]. Conventional chemotherapy can inhibit the growth of the tumour in the breast and can kill cancerous cells. This comes with the side effects- nausea and vomiting, alopecia, early menopause, fatigue, mouth and throat sores, weight gain, nail weakness and memory problems [8]. Targeted therapies such as monoclonal antibodies (Transtuzumab) against specific genes and receptors involved in molecular pathways although show a significant digression, studies indicate that prolong treatment do not have much effects on the disease itself [6]. Phytochemicals are one of the best sources for preventing the uncontrollable proliferation of cancerous cells. The current study explores the utility of major herbal based compounds as probable inhibitors against various drug targets of breast cancer by computational virtual screening and preliminary in vitro assays.

2. MATERIALS AND METHODS

2.1. Identification of potential drug targets involved in Breast Cancer pathways

Literature survey was carried out to study the important molecular pathways involved in Breast Cancer proliferation

and also for selection of active receptors present in MCF-7 Breast Cancer cells. Some of the screened receptors include Glucocorticoid agonist, kinase domain of epidermal growth factor receptor (ErbB2) [9] and ligand binding domain of the human nuclear receptor RXR alpha. The genes involved in these receptors were found to be over expressed in Breast Cancer and hence were considered to be important therapeutic targets.

2.2. Selection of receptors

Some of the important drug targets found based on literature survey were Glucocorticoid agonist, kinase domain of ErbB2 [9], ligand binding domain of the human nuclear receptor RXR alpha. The three dimensional structures were retrieved from RCSB-PDB. PDB ID of glucocorticoid receptor was 1M2Z [10] whose R-value was 0.267 and R-free value was 0.267. The PDB ID of ErbB2 was 2A91 [11] whose R-value was 0.228 and R-free value was 0.264. The structural features of other receptors are illustrated in Fig.1.

2.3. Selection and computational screening of Phytoligands

Phytoligands present in various plant sources were listed based on its inhibitory potential and geographical location after carrying out the extensive literature survey. Some of the chemical, pharmacological, pharmaceutical data about phytoligands were studied using the DrugBank [12] database. The 3D chemical structures of screened phytoligands were retrieved from NCBI PubChem [13]. The sdf files were retrieved from PubChem and were converted to pdb format by OpenBabel [14] software. PreADMET tool was used to predict the best lead molecules based on the drug likeness, ADME properties and toxicity.

2.4. Molecular docking of screened phytoligands with receptors

AutoDock 4.2 [15] was used for docking studies of the screened phytoligands with MCF-7 receptors. The PDB file formats for phytoligands and receptors were prepared. AutoDock was executed to simulate the real time molecular interactions of the macromolecule and ligand. The docking (log) file was read and the minimum energy value was determined. The interaction between receptor and ligands were displayed and the best conformations were selected. The best docked results were analysed and the phytoligands with least binding energy value were selected and further considered for in vitro studies.

2.5. In vitro testing of Apigenin against MCF-7 cells

Dulbecco's Modified Eagle's Medium (DMEM) was prepared and was sterilized using autoclave. The cells were cultured using this media. Once the cells reached confluence stage, the tripsinization was performed by adding trypsin-EDTA which led to the detachment of cells. The culture was centrifuged at 1500 rpm. The cell suspension was obtained by dissolving the pellet in the media. The cryovial containing the cell suspension and freezing mixture was prepared and kept in the slow cooling device. The cell suspension was returned to the normal state from the frozen state by keeping the vial at room temperature. Based on the computational virtual screening Apigenin was identified as the best herbal lead molecule. The pure form of this chemical was obtained from Natural Remedies, Bangalore, India. This compound was used for the further assays. Trypan blue exclusion assay was carried out to determine cell viability. MTT assay was carried out for different drug concentration and percentage viability of the cells was calculated. From this IC_{50} (half maximal inhibitory concentration) value was determined graphically.

3. RESULTS AND DISCUSSION

3.1. Selection of suitable Breast cancer cell line

Several cell lines for breast cancer such as AU565, BT-20, MCF-7, MDA-MB-231, SkBr 3 and T47-D were researched and studied for their properties [16]. MCF-7 cell line was selected for this study based on the major receptors that are present and their involvement in various cancer pathways. MCF-7 cell line is a standard cell model employed for breast cancer studies.

3.2. Screening of receptors and identification of molecular pathways present in MCF-7 cell line

Some of the most important receptors present in the MCF-7 cell line that play significant role in cancer progression and development are estrogen alpha [17], androgen receptor, progesterone receptor, glucocorticoid receptor [18], C-X-C chemokine 4 [19], Epidermal growth factor (ErBb2) [20], IGF-1 [21], Insulin receptor [22], Neuropeptide YY1 receptor [23] and TGF beta-2 receptor [24] (Fig. 1) Many significant signalling pathways and functions of the receptors present in those pathways were studied by Therapeutic Target Database and KEGG Pathways. The amino acid sequences from Uniprot database were retrieved and their three-dimensional structures were obtained from PDB. The number of amino acids present in the protein structure, number of polypeptide chains, its resolution, R-factor and type of secondary structures present were studied.

3.3. Identification and screening of phytoligands with anticancer properties

Based on the literature review 54 ligands were screened for computational virtual screening. Their IUPAC names, molecular formulae, sources, common names and receptors against which they are potent were identified. Different indigenous plant sources were considered based on the

availability [25, 26].The three-dimensional structures of these ligands were retrived from PubChem [13] (Fig 2).

Fig. 1. The crystal structures of four important receptor visualized by PyMol. (a) PDB Structure of estrogen receptor Alpha (PDB ID: 1A52). This receptor is made up of 2 polypeptide chains A and B and consists of 258 amino acids. Gene names are ESR1, ESR and NR3A1. It is majorly made up of alpha helices (64%) and random coils and contains very few beta strand secondary structures (3%). The resolution of this X-Ray crystallography structure is 2.8 Å. The R-Value was found to be 0.223. (b)PDB structure of kinase domain of ErBb 2 receptor (PDB ID: 3PP0). This receptor is made up of 2 polypeptide chains A and B and consists of 338 amino acids. Gene nAmes are ERBB2, HER2, MLN19, NEU and NGL. It contains 33% helices and 17% beta sheets. The resolution of this X-Ray crystallography structure is 2.25 Å. The R-Value was found to be 0.189. (c)The PDB structure of human TGFβ2 receptor (PDB ID: 1TFG). This receptor consist of one polypeptide Chain A and consists of 112 amino acids. It contains 21% helices and 41% beta sheet. The resolution of this X-ray crystallography structure is 1.95Å. The R-Value was found to be 0.194.(d) The PDB structure of human nuclear receptor RXR alpha (PDB ID: 1LBD). This receptor is made up of one polypeptide chain A and consists of 282 amino acids. Gene names include RXRA and NR2B1. It contains 55% helices and 2% beta sheets. The resolution of this X-Ray crystallography structure is 2.7Å. The R-Value was found to be 0.230.

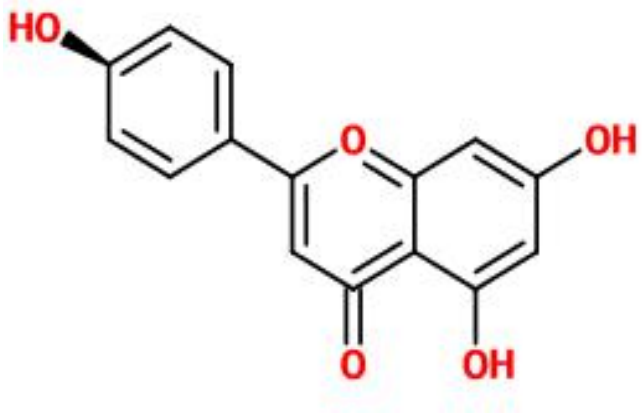

Fig. 2. Structure of Apigenin, Molecular formula: $C_{15}H_{10}O_5$ Molecular weight: 270.2369, IUPAC: 5, 7-dihydroxy-2-(4-hydroxyphenyl) chromen-4-one.

3.4. Drug likeness studies

Lipinski's Rule of Five, Lead-like Rule, CMC Rule, MDDR-like Rule and WDI like Rule were used to predict the drug likeliness for the phtoligands. Out of these five rules, a compound has to qualify minimum three rules for it to be selected as a lead molecule. After ADMET and drug likeness studies, 33 phytoligands qualified and were selected for further studies. The final selected molecules are shown in Table 1.

TABLE 1: Prediction of drug likeliness and ADMET of the best herbal ligands screened by Computational virtual screening using PreADMET tool.

Active compound	Lipinski's rule (rule of five)	Lead-like	CMC rule	MDDR-like rule	WDI-like rule	Minimum 3 rules qualified
Apigenin	Suitable	Suitable if its binding affinity is greater than 0.1 μM	Qualified	Mid-Structure	In 90% cut off	Yes
Biochanin A	Suitable	Suitable if its binding affinity is greater than 0.1 μM	Qualified	Mid-Structure	In 90% cut off	Yes
Chrysin	Suitable	Suitable if its binding affinity is greater than 0.1 μM	Qualified	Mid-Structure	In 90% cut off	Yes
Eriodictyol	Suitable	Suitable if its binding affinity is greater than 0.1 μM	Qualified	Mid-Structure	In 90% cut off	Yes
Isoliquiritigenin	Suitable	Suitable if its binding affinity is greater than 0.1 μM	Qualified	Mid-Structure	Out of 90% cut off	Yes
Lithocholic Acid	Suitable	Violated	Qualified	Mid-Structure	In 90% cut off	Yes
Myosmine	Suitable	Violated	Not Qualified	Mid-Structure	In 90% cut off	Yes
Naringenin	Suitable	Suitable if its binding affinity is greater than 0.1 μM	Qualified	Mid-Structure	In 90% cut off	Yes
Quercitin	Suitable	Suitable if its binding affinity is greater than 0.1 μM	Qualified	Mid-Structure	In 90% cut off	Yes

3.5. Computational prediction of ADME properties

The absorption, distribution, metabolism and excretion (ADME) are the most fundamental part of pharmacological studies of lead molecules. Using PreADMET tool Absorption, Distribution, Metabolism and Excretion studies of the 54 listed ligands were predicted. After the ADME studies, 27 compounds were selected based on HIA (70-100 %), in vitro skin permeability (-1 to -5 cm/hour), in vitro blood barrier penetration (+90 %) and many other criteria.

3.6. Computational prediction of toxicity

Under this category, Ames test [27] and Rodent Carcinogenicity assay values were computationally assessed [28]. Most of the phytoligands selected were mutagens but non-carcinogenic in mouse models.

3.7. Docking studies

Docking studies were carried out with AutoDock v 4.2 [29] for screening the best ligands. Various parameters such as number of hydrogen bonds, number of interacting residues and length in angstrom, residues involved in hydrogen bonds, covalent bonds and hydrophobic Interactions were analyzed. Those complexes which had maximum number of hydrogen bonds with minimum binding energies were selected. AutoDock performs molecular docking by pre-calculating energy grids around a site of interest on the target. The interaction between receptors and ligands were displayed and the best conformations were selected. The interacting residues were labelled. The hydrogen bonds formed between ligand and receptor were visualized and saved. Apigenin was selected to dock with 20 receptors present in MCF-7 cell lines. The best docked conformations resulted by the interaction of Apigenin and selected cancer receptors are shown in Table- 2. Out of these 20 docked structures, 2 docked structures had the minimum binding energies. Apigenin docked with Glucocorticoid agonist receptor demonstrated a binding energy of -6 kcal/ mol (Fig 3a) and with Human Transforming Growth Factor Beta 2 showed a binding energy of -4.92 kcal/mol (Fig 3b). Apgenin is a phytoligand present in various herbal sources such as Petroselinum crispum (Parsley), Apium graveolensvardulce (Celery), Matricaria chamomilla (Chamomile tea) [30]. Apigenin is a less-toxic and non-mutagenic flavonoid. The formulated Apigenin powder that was obtained was of 97% purity.

3.8. Trypan blue exclusion assay for cell viability

In this assay, live cells that possess intact cell membranes exclude certain dyes, such as trypan blue, eosin, or propidium, and a translucent ring appears around the cells whereas dead cells take up the stain and appear opaque under the microscope. In this test, a cell suspension is simply mixed with dye and then visually examined to determine whether cells take up or exclude dye.

TABLE 2: The binding potential of Apigenin towards various selected receptors predicted by molecular docking. The best docked conformations was selected based on minimum energy and number of interacting residues

Receptor name	Binding energy (kcal/mol)	No. of hydrogen bonds	Amino acid residues interacting with the receptor
Human TGF beta 2	0.0	1	CYS-44, PHE-43, TYR-39, ALA-41, MET-104
Kinase domain of ErbB2	0.0	3	ASP-950, LYS-765, TYR-772, MET-979, GLU-975, GLU-971, PRO-967
Glucocorticoid agonist	-6.0	1	GLN-615, ASN-630, ILE-628, PRO-625, CYS-622
pH domain of human Akt3 protein kinase	-2.85	2	PRO-67, ASN-21, TRP-22
Neuropeptide Y1 receptor	0.0	1	ILE-268, ALA-271, CYS-275, ASN-318, VAL-126, LEU-82, VAL-89, ASP-86
IGF1 receptor kinase A	-3.1	2	THR-1127, MET-1126, ARG-1109
ILGFR domains 1 to 3	-3.55	1	CYS-277, GLU-276, GLN-275, GLU-242
Insulin receptor domains 1 to 3	-2.7	0	ASN-90, PHE-89, ASP-142
1 to 3 domains of ErbB2	0.0	1	CYS-90, LEU-86, ILE-93, THR-175, VAL-176, MET-180, TYR-172, LEU-179
TK binding domain of ErbB2	-4.7	2	ASN-38, THR-6, TYR-282, GLN-85, ASP-9, LEU-415, LEU-292, SER-442
IGF1 receptor kinase domain	-3.14	0	ARG-1042, GLU-1046, ILE-1045
Ectodomain of human GHRH	0.0	2	THR-75, ALA-91, TRP-73

3.9. MTT assay

The MTT assay was carried out at Leads-Clinical Research and Bio services Pvt Ltd, Bangalore, India. The IC_{50} value for Apigenin against MCF-7 cells is found to be 35µg (Fig. 4b). The IC_{50} values range from >2000µg/ml to about 8µg/ml for synthetic and natural Apigenin respectively. The IC_{50} value also depends on the conformation of the receptor and also the phytoligand that binds. Hence for each different conformation binding energy is different and therefore the efficacy of the drug. The morphological changes of cells were seen after treatment with different concentrations of Apigenin 48 hours post treatment (Fig 4a). The MTT assay is a colorimetric assay for assessing cell viability. Tetrazolium dye assays can be used to assess cytotoxicity or cytostatic activity of potential bioactive compound and toxic materials. MTT assays are typically performed in the dark as the reagent is sensitive to light. Absorbance values that are less than the control cells designate a decrease in the rate of cell proliferation. However, higher absorbance rate shows an increase in cell proliferation. Seldom, a raise in proliferation may be offset by cell death which indicates cell death may be inferred from morphological changes [23].

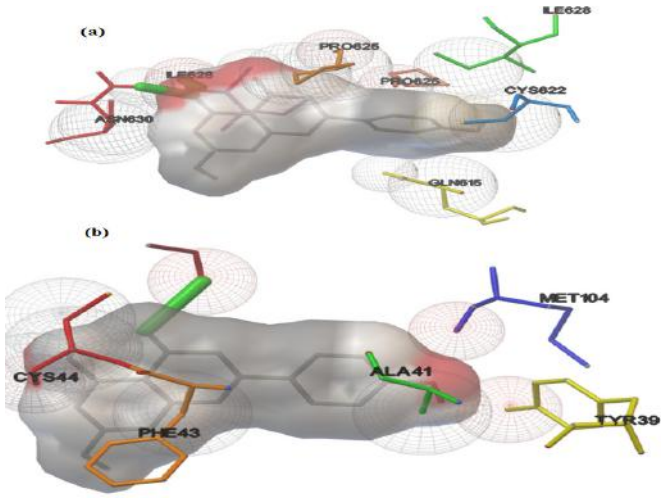

Fig. 3. The Binding potential of Apigenin against two probable receptors predicted by molecular docking (b) Interaction between Apigenin and Glucocorticoid receptor stabilized by two hydrogen bonds (binding energy: -6.0 kcal/mol) Interaction between Apigenin and Human TGF Beta2 receptor stabilized by a hydrogen bonds (binding energy: -4.92 kcal/mol)

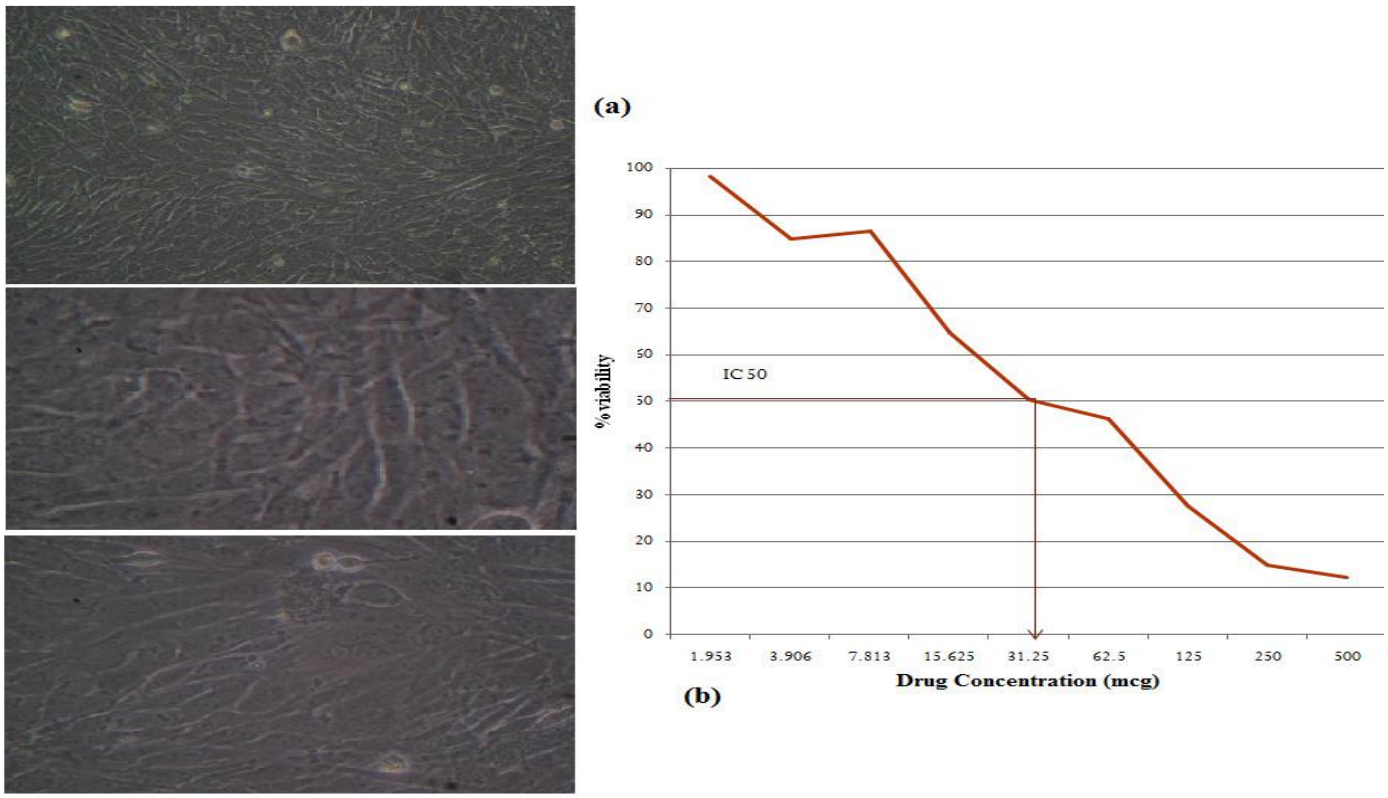

Fig. 4. Inhibitory activities of Apigenin against MCF-7 cell lines analyzed by in vitro assay (a) Morphological changes of MCF-7 cell lines cells were seen after treatment with different concentrations of Apigenin 48 hours post treatment by MTT assay (b) Determination of IC_{50} for Apigenin against MCF-7 from percentage viability and concentration of Apigenin (µg) was found to be 35 µg.

4. CONCLUSION

The current study concludes that computational virtual screening is one of the best approaches to screen natural therapeutics against various receptors of breast cancer cell lines and phytoligands play vital role in inhibiting the major drug targets of various cancerous cells. In vitro studies suggested that Apigenin can be used as one of the therapeutic remedies against various breast receptors. The current study paves profound insight for further experimental analysis and has high future scope.

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